Coupled Physiology and Genomic studies of Isolate 1L, a sulfate-reducing bacterium from the Prairie Pothole Lake Region

A Senior Research Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in Microbiology in the undergraduate colleges of The Ohio State University

By

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Abstract

The prairie pothole region of North America, covering approximately 750,000 km^2, contains millions of small lakes that are important systems for regional carbon and sulfur cycling. Prairie pothole lake (PPL) sediment pore waters contain some of the highest dissolved organic carbon concentrations ever measured in freshwater ecosystems. Sediment and pore water samples from the PPLs of North Dakota were used to anaerobically enrich for sulfate reducing bacteria (SRB) and fermentative microorganisms. Given the high sulfate concentrations characteristic of PPL pore waters, we suspect that SRB play an important role in coupled carbon and sulfur cycling, while fermenters degrade more complex carbon substrates into labile compounds. A range of medias, enrichments, and methods were used to cultivate and isolate representative microorganisms, with growth monitored through assays and epifluorescence microscopy. As a result, isolates 1L (a SRB growing with sulfate and lactate) and YPD1 (a fermenter) were obtained. A BLAST search of the 16S rRNA sequence from 1L revealed that it was closely related to *Desulfovibrio magneticus*. While Desulfovibrio sp. 1L tolerated elevated sulfide concentrations (up to 6mM), salinity of growth media was a strong constraint on growth. The genome of YPD1 showed the isolate to be *Proteinoclasticum ruminis*. Future studies will continue to analyze the genomes of these isolates and determine their role in PPL sediments through planned experimentation. Studying the interactions of these isolates with other key microbes in the community such as methanogenic archaea will provide insight into key processes driving carbon and sulfur cycling in the environment.

Introduction

Natural and anthropogenic wetlands account for 7% of Earth's land surface and are responsible for 30-40% of global emissions of methane. Natural wetlands can act as a net sink of carbon depending on wetland type, age, and location as well as the prevailing climate and environmental conditions (Pester, Knorr, Friedrich, Wagner, & Loy, 2012). Organic carbon degradation in wetlands is catalyzed by different functional guilds of aerobic and anaerobic microorganisms, whose competition for electron donors, or syntrophic cooperation, determines how much of the carbon loss from wetlands proceeds through the emission of carbon dioxide or the more potent greenhouse gas methane. Because sulfate-reducing microbes (SRM) couple sulfate dissimilation with heterotrophic carbon degradation or carbon dioxide fixation, sulfate reduction directly influences the carbon cycle in wetlands (Pester et al., 2012).

The Prairie Pothole Region is a natural, freshwater wetland ecosystem that contains millions of small lakes (PPLs) and covers about 750,000 km^2 in North America (Figure 1). Pore waters from these lake sediments contain the highest concentrations of dissolved organic carbon (DOC) ever measured in freshwater bodies, and while lake waters are oxygenated, the underlying sediments are mostly anaerobic. Additionally, the region is characterized by high levels of sulfur (S) species existing in three main pools: solid-phase pyritic S, reduced organic S, and oxidized S. No significant seasonal variation is evident for total S, but S speciation shows a seasonal response (Zeng et al., 2013)(Figures S2 and S3).

Particularly in lake P1, during the summer-fall transition the reduced S pool grows significantly while the amount of oxidized S species diminishes (Figure S3). These observations provide evidence for the importance of microbial activity in the recycling of

sulfate in the PPL system and their influence on the carbon cycle. The sulfur cycle is unlike other cycles as sulfur is not lost to the atmosphere in high amounts like in the nitrogen cycle with nitrogen gas. It has an under recognized role in the cycle of carbon in determining the form in which carbon is lost. SRM are energetically favored in the competition for substrates with microorganisms involved in the methanogenic degradation pathways, resulting in a considerable diversion of the carbon flow from methane to carbon dioxide (Pester et al., 2012). Methanogenic microbes are responsible for consuming fermentative substrates in the environment such as acetate and lactate, which are important for the growth of SRM (Figure S4).

Understanding processes that may control S speciation in PPLs including sulfate reduction rates, viral influence, and other environmental factors, can help us determine potential constraints on mercury methylation and the activity of methanogens. It is unclear how global climate change will affect or be affected by microbial processes in freshwater wetlands and by studying these microbial interactions, we can gain insight into the impact of SRM on global sulfur and carbon cycles. Additionally, we can further understand why sulfate reduction is considered a critical anaerobic degradation process and how microbes act as recycling mechanisms to maintain a balanced S cycle, avoiding the rapid depletion of sulfate pools (Pester et al., 2012).

To better understand sulfur cycling in PPL regions, I worked to isolate both SRMs and fermentative bacteria from PPL sediments (Figure 2). This effort was successful, resulting in pure cultures of a sulfate-reducer and a fermenter. This subsequently allowed me to study their metabolism to gain further knowledge of their role in PPL environments.

Methods

Experimental Design

Freshly collected sediment cores from Prairie Pothole Lakes were sectioned into 4-cm intervals at Ohio State's Byrd Polar Research Center. Sediment sub-samples (~ 1g) from a depth of 16-20 cm in lake P1 were placed in anaerobic enrichment bottles in a defined, minimal, basal bicarbonate media denoted Freshwater media (FW), amended with approximately 1 mL of dissolved organic carbon (DOC) extracted and filtered from lake P7, and 20mM sodium sulfate (Figure S1). Sediments were added in the anaerobic environment of the COY anaerobic chamber, which is maintained with a 2-3% hydrogen atmosphere balanced with nitrogen. The serum bottle was capped with a thick butyl rubber stopper and was stored in a dark incubator at 37ºC.

Monitoring Growth

The enrichment's progress was monitored via the HACH colorimetric sulfide assay (HACH Loveland, CO cat. 2244500). A standard sulfide curve was created for use in calculating the micro molar amounts of sulfide measured in enrichment samples. Equation 1 was derived from this curve and used for these calculations.

Eqn.1 $$y = 0.0128x - 0.0359$$

A spectrophotometer reading at 650nm was used for measuring samples. In addition, bright field and epi-fluorescence microscopy was utilized in order to monitor the overall diversity and growth of the culture and to assess the need for transfer into new media.

Optical density readings taken at 600nm were also a viable method to confirm growth of cultures via light scattering and turbidity.

Isolation Techniques

Inoculum from cultures showing signs of positive growth was used to inoculate solid isolation media held in plastic pipet bulbs (Figure 3). Solid isolation media was designed to precipitate black iron sulfide to indicate growth in the chosen media (Figure S1). Freshwater media was chosen, inoculated with culture (10%), amended with 20mM of an electron donor and acceptor, drawn up by flexible plastic pipet bulbs, and sealed inside by bending and zip-tying the necks to decrease the chance of oxygen reaching the media. The bulbs were stored in the 30ºC incubator inside the anaerobic chamber.

The advantage to using these containers has to do with the colony extraction process when growth is present. The formation of black iron sulfide precipitates is associated with localized regions of cell growth in the media and indicates when extraction of colonies is necessary. Sterile razor blades, scalpels, and petri dishes were used to cut open the sides of the bulbs, dissect and section the media into workable pieces, and extract individual colonies to be placed back in liquid media. The plastic of the pipet bulbs is easy to see through and easily cut when needed, decreasing the chance of cross contamination of the colonies. Sterile technique was practiced to the best of its ability with ethanol washes of equipment between extraction of each colony and the procedure carried out in the anaerobic chamber.

Assessment of Isolate Purity

The purity of isolate 1L was assessed through microscopy, DNA extractions, and 16S rRNA gene analyses. Bright field microscopy was used to judge the similarity of cellular morphology before DNA was extracted for analysis. This analysis was necessary to

determine if contamination was present, indicated by another sequencing signal in the sample.

Characterization

Phylogenetic and Taxonomic Placement

To determine the phylogenetic placement of isolates, their 16S rRNA sequences were aligned to the SILVA database in order to find close relatives and construct a maximum likelihood phylogenetic tree. 16S rRNA sequences of related neighbors and the isolate were compiled and aligned to produce a maximum likelihood phylogenetic tree in the program Seaview. The PhyML tree program was run with 100 bootstraps and rooted with an appropriate organism. The resulting tree was used to confirm placement and illustrate relatedness of the isolate to other organisms.

Salinity Tolerance

The tolerance of isolate 1L to sodium chloride (NaCl) was tested with various concentrations of salinity and against the tolerance of a *Desulfovibrio vulgaris* strain. Concentrations of NaCl ranged from 0 – 130g/L for 1L and 0 – 60g/L for *D. vulgaris*. Anaerobic tubes of FW media amended with 20mM sodium sulfate, 20mM sodium lactate, and the assigned concentration of NaCl were inoculated with the appropriate microbe. Each concentration was performed in triplicate and a tube lacking artificial saline used as the control. A growth curve spanning over 315 hours was determined with optical density readings taken at 600nm using a portable HACH spectrophotometer and tubes stored in an incubator set at 30ºC in between readings.

Sulfide Tolerance

The minimum inhibitory concentration of sodium sulfide for isolate 1L was determined through a series of growth tests. Sulfide concentrations ranging from 1mM-6mM were added to FW media at the time of inoculation. Spectrophotometer optical density readings at 600nm were taken over a period of 200 hours.

Substrate Utilization

Various substrates including thiosulfate, butyrate, acetate, formate, ethanol, and propionate were added to FW media and inoculated with isolate 1L (10%) to determine the range of sources it utilizes. Cultures were stored in a 30ºC incubator and visualization of turbidity used to judge the success of growth.

Epifluorescence Microscopy

The Nikon Eclipse Ci-S/Ci-L microscope was used to view and capture images of cultures and isolates. To help distinguish between living and non-living matter under the microscope, the fluorescent DNA-binding dye SYBR Gold was used to stain cells. The appropriate concentration of SYBR Gold was added to roughly 1mL of culture and let stand in a dark area for an hour. Slides were then prepared and stored in the dark before viewing.

Viral Induction

FW media was inoculated with isolate 1L (10%) in a series of tubes. Using a predetermined growth curve, various chemical stressors were added near the end of the exponential phase. The stressors used were mitomycin C, a copper chloride solution of ~0.6mM, a pH adjustment from 7.75 to 5.93 using hydrochloric acid, and the addition of a high concentration of sodium sulfide (100mM). Cells were stressed to encourage viral

activity and a kill curve used to determine their presence. Optical density readings before and after induction were taken by a portable HACH spectrophotometer at 600nm. This protocol follows one similar to the Motlagh et al. procedure (Motlagh, Bhattacharjee, & Goel, 2015).

Anaerobic media and stock preparation

Media, stock solutions, and any other materials added to cultures was prepared and driven anaerobic. For media, ingredients were added and mixed into deionized water using a stir plate (Figure S1). A gaseous mixture of 95% nitrogen gas and 5% carbon dioxide gas was pumped through the media for ~45 minutes to ensure oxygen was replaced. The pH was read and adjusted accordingly using hydrochloric acid to be a neutral 7. The headspace of the container was degassed as well and then taken into the anaerobic chamber to be distributed as needed before autoclaving. A similar procedure was used for preparing stock solutions of various materials. For materials not able to be autoclaved, the solution was filtered through a 0.45 μm filter into a sterile container.

Genomic Analyses

DNA Extraction

The extraction kit used for gDNA extraction was the PowerSoil® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA). DNA of samples was extracted using conserved universal 16S bacterial primers (27F, 1492R) and amplified via PCR. Thermal cycler settings were as follows.

1. 95ºC for 5 min
2. 95ºC for 45 sec
3. 56ºC for 1 min

4. 72ºC for 1 min 15 sec
5. Repeat steps 2-5 for 30 cycles
6. Hold at 4ºC

To verify the product length was correct (~1,450 bp) for the 16S rRNA gene, a sample of the extracted PCR product was run on a PCR gel. After confirmation, a PCR cleanup kit was used to prep for Sanger sequencing. This was the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). A clean PCR product was sent to the Plant-Microbe Genomics Facility (PMGF) of the Ohio State University where chromatograms and fasta files were generated to determine the purity of samples and their identity.

Genomic gDNA Preparation and Sequencing

A sample of culture was taken and spun down at top speed (18,000g) for 20 minutes at 4ºC, and supernatant discarded, pelleted DNA/cellular debris added to a lysis tube, and used for extraction following the recommended guidelines. The kit used for extraction of a low biomass isolate was the ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research Corp., Irvine, CA). The only deviation from the manufacturer's protocol was the first step. Instead of using provided bead beater tubes, a replacement was made using the following reagents and added to the DNA pellet: lysis buffer, 10uL RNAse (5mg/mL), 20uL of 10% SDS (wt/vol), 30uL lysozyme (100mg/mL), 5uL proteinase K (20mg/mL), ~200mg 0.1mm zirconium beads. The self-made bead tubes were incubated at 37ºC for 20 minutes and the remaining instructions of the protocol followed. Results were quantified with a Qubit Fluorometer and sent to the Genomics Shared Resource (GSR) facility of the Ohio State University for genome sequencing performed using the Illumina HiSeq2500 sequencing platform.

Genome Annotation and Analysis

The server on behalf of the Wrighton Lab performed automatic annotation of genomic sequences received from the GSR facility. Another tool on the server for computing similarities across multiple genomes called ITEP: an integrated toolkit for exploration of microbial pan-genomes was also used.

Results and Discussion

Isolates from Enrichments

Isolates denoted YPD1 and 1L resulted from environmental enrichment attempts. Their physiology and characteristics were explored, revealing YPD1 to be a fermentative organism and 1L a sulfate reducing bacterium (SRB).

Isolate YPD1 was obtained from a lake P1 sediment enrichment collected at a depth of 16-18cm using freshwater media (FW) and dissolved organic carbon (DOC) concentrated from P7 lake water. After growth in sodium propionate-amended liquid media, the culture was spread onto anaerobic plates of YPD media and stored in an anaerobic chamber at 40ºC. When growth was observed, colonies were picked from the plates and transferred back into liquid YPD media. Of the colonies harvested, one resulted in a pure culture and was sent to the Joint Genome Institute of the United States Department of Energy (JGI). Genome analysis confirmed YPD1 as a strain of *Proteiniclasticum ruminis*, a fermenter, closely related to one described strain, *P. ruminis* DSM 24773 (Zhang, Song, & Dong, 2010). A brief analysis of the genome suggests the potential uptake of complex sugars as carbon sources for fermentation as well as the

presence of an acetate kinase gene responsible for the generation of acetate as a fermentation product.

Isolate 1L was obtained from a lake P1 sediment enrichment collected at a depth of 16-18 cm using FW, 20 mM sodium sulfate, and DOC concentrated from P7 lake water. Following growth in FW media amended with DOC, this culture was used to inoculate isolation media in pipet bulbs as described in the methods section. One of the original 21 colonies extracted from the pipet bulbs survived transfer into liquid FW media amended with sulfate and lactate, resulting in a pure culture of an unidentified isolate of genus *Desulfovibrio* as confirmed by 16S rRNA analysis. Genome data suggests its closest neighbor is *Desulfovibrio magneticus*. The taxonomy classification according to the SILVA database for isolate 1L is:

Bacteria→ Proteobacteria→ Deltaproteobacteria→ Desulfovibrionales→ Desulfovibrionaceae→ Desulfovibrio

Characterization of Isolate 1L

Isolate 1L is classified as a flagellated rod-shaped SRB growing optimally in media containing sulfate as an electron acceptor and lactate as an electron donor. Using the microscope program Nikon NIS Elements, 1L was found to be an average length of 1.6μm (Figure 4). Physiology was classified through a series of experiments for various environmental stresses characteristic of PPL environments. These experiments included salinity tolerance, sulfide tolerance, utilization of various organic and inorganic substrates, cellular morphology by microscopy, and induction for viruses.

A growth curve was performed in triplicate using concentrations of sodium chloride (NaCl) ranging from 0-130 g/L and *Desulfovibrio vulgaris* as a representative organism for comparison. The specific growth rates for each curve were calculated, averaged, standard deviation determined, and plotted against salinity (Figure 5). The data illustrates salinity of growth media exists as a strong constraint on growth for the isolate, as biomass did not increase at salt concentrations above 30 g/L.

Given the environmental conditions of the PPLs and other supporting data, we suspected 1L to have a tolerance to elevated sulfide concentrations of 2-3mM (Figure S2) (Zeng, Arnold, & Toner, 2013). The minimum inhibitory concentration of sulfide for 1L was determined using a series of cultures amended with increasing concentrations of sodium sulfide and growth measured by optical density readings. Performed in triplicate, specific growth rates were calculated, averaged, standard deviation determined, and plotted against the corresponding concentration of sulfide (Figure 6). Due to the degree of inconsistency as shown by standard deviation, some results should be repeated to determine a defined inhibitory concentration. The growth rate of 1L decreases with an increase in sulfide, but appears to plateau around a specific growth rate of 0.015. Although elevated sulfide concentrations clearly act as a stressor on 1L, the isolate still grows under the stress of elevated sulfide and tolerates ~3 times the amount it encounters in the PPL environment.

Isolate 1L does not appear to have the metabolic diversity common among sulfate reducers. Various substrates were added to cultures of 1L to determine its metabolic plasticity (Table 1). The ideal combination of electron donor and acceptor, lactate and sulfate, was used as the control. It is important to note the culture containing thiosulfate as

an electron acceptor grew at a faster rate than the control, primarily because thiosulfate is considered an activated form of sulfate. It was later found 1L possesses a rhodanese enzyme (also called thiosulfate sulfurtransferase) in its genome that allows for the conversion of thiosulfate to sulfite. The activity of the rhodanese enzyme eliminates the necessary step of expending ATP to activate sulfate by the enzymes ATP sulfurylase and APS reductase. Avoiding this step increases the reduction rate and thusly the growth rate of the organism. When using ethanol as an electron donor and carbon source, it progresses through the classical pathway of fermentation from alcohol, to aldehyde, to fermentation product. Evidence in the genome supporting this claim is the presence of various alcohol dehydrogenases.

Isolate 1L was induced with a variety of cellular stressors to detect the presence of bacteriophage (Figure 7). Cells were induced approximately 42 hours after inoculation during the mid-log phase of growth. The data did not show evidence of bacteriophage being present as a drop in optical density was absent. These results contradict genomic evidence later confirming the presence of phage in the genome as determined by the VirSorter program for identifying phage sequences. The evidence of phage may have not appeared in experiments due to cells not being stressed enough or other unknown reasons.

Genome Analysis

After genome sequence data was obtained, assembled, and annotated, features of interest were identified. The draft genome of isolate 1L resulted in a predicted ~3900 genes across 233 scaffolds. There were 7.6 million total base pairs sequenced with an average sequence length of 13,766 base pairs. The following is a preview into some of the key genomic features of isolate 1L.

Clustered regularly interspaced palindromic repeats (CRISPRs) are loci encompassing several short repeats functioning as an adaptive microbial immune system. Several types of CRISPR-associated proteins (Cas) are encoded by *cas* genes located in the vicinity of CRISPRs (Morais-Silva et al., 2014). Their identity, operon organization, and spacing are essential to their classification into CRISPR/Cas systems, which can be used to reveal evolutionary relatedness among other CRISPR containing organisms. Isolate 1L has evidence in its genome of genes *cas1, cas2, cas3, cas4, cas5, csd2,* CRISPR-associated protein tm1801, and an HD domain-containing protein. There exist six potential CRISPR systems confirmed by the CRISPRFinder program, a web tool designed to identify repeat and spacer sequences (Figure 8)(Grissa, Vergnaud, & Pourcel, 2008). The program VirSorter, a pipeline designed to mine microbial draft genomes for viral signals, also detected potential phage proteins. Genes 68-75 on scaffold 20 were designated as phage. Following a blastp search of each gene's amino acid sequence, gene 69 hit to a possible regulatory protein FmdB, thought to regulate the expression of FmdA, a formamidase. The proposed mechanism by which FmdA is induced by amides and repressed by ammonia remains to be determined (Wyborn, Mills, Williams, & Jones, 1996).

Dissimilatory sulfate reduction is the ability of microorganisms to reduce multiple oxidized sulfur species resulting in the production of energy and hydrogen sulfide. The three enzymes that regulate sulfate reduction are ATP sulfurylase, APS reductase, and bisulfite reductases or *dsr* genes (Figure 10). *Dsr* genes serve as evolutionary markers of sulfate reduction and can be found in all sulfate reducing organisms. Isolate 1L has all three of these vital enzymes, including both A and B subunits of the *dsr* gene. The relatedness of 1L's *dsrA* genes to those of other SRB is shown in figure 9, which compares

the translated amino acid sequences of all the *dsrA* genes. The closest related *dsrA* sequence to isolate 1L is *Desulfovibrio vulgaris* (accession number WP_010937709.1).

In addition to the traditional enzymes involved in sulfate reduction, 1L also contains rhodanese. This enzyme is typically associated with the activity of converting cyanide to thiocyanate as a defense when cells are exposed to harmful levels of cyanide. Rhodanese is also known as thiosulfate sulfurtransferase and has been shown in previous literature to convert thiosulfate to sulfite (Yoch & Lindstrom, 1971). Sulfite is a reduced form of sulfate and is reduced further to hydrogen sulfide with bisulfite reductases.

The PPL sediment environment is anoxic with the exception of some mixing of aerobic lake waters in the top 5cm of lake sediment. While the depth of sediment used to obtain a culture of isolate 1L was 16-18cm below the sediment-water interface, the nature of these bacteria suggests they have adaptations for adjusting to and managing oxidative stress. Having the ability to sense and respond to environmental stimuli is critical for maintaining elements like iron-sulfur proteins that are sensitive to oxygen. The genome of isolate 1L has elucidated several methods for coping with oxygen in its environment. Genes known for oxidative stress protection that exist in the genome are super-oxide dismutase, endonuclease III and IV, thioredoxin, glutaredoxin, rubrerythrins, and peroxiredoxin (Cabiscol & Ros, 2000; Morais-Silva et al., 2014). Its closest neighbor, *Desulfovibrio magneticus*, contains genes that code for an organelle called a magnetosome. Its function allows the bacterium to swim toward magnetic fields and is a way the bacteria respond to their environment. Our isolate does not show evidence for these genes in the genome so other regulators for movement must be expressed.

Nitrogen typically exists as organic matter in the environment and is an essential element for microbial growth. Within sediments some microbially driven processes (denitrification and anammox) result in the net removal of nitrogen from the environment, while others (dissimilatory nitrate reduction to ammonium, DNRA) do not (Sediments, Smith, Nedwell, Dong, & Osborn, 2007). In order for microbes to access nitrogen, it must first be fixed from nitrogen gas to another form of nitrogen. The DNRA genes found in 1L supports the inference of a key role for this microorganism in the PPL nitrogen cycle. In the PPL environment, sulfate reduction occurs at a fast rate resulting in a rapid turnover of sulfate. Because sulfate is not always an available electron acceptor in the environment, we propose the excess reductant is disposed of on nitrite instead (Figure 10). The presence of genes for cytochrome c nitrite reductase supports our hypothesis. Another benefit to the proposed system is the fixation of nitrogen in the environment perhaps for other microorganisms inhabiting PPLs.

Another powerful analysis tool used on the server was the program ITEP (An **I**ntegrated **T**oolkit for **E**xploration of microbial **P**an- genomes), which was used to further analyze a constructed database of *Desulfovibrio* sp. genomes. A group of eleven organisms including 1L was constructed by pulling genbank files from the NCBI database. The program uses genomic data from the files and computes similarities to externally defined domains, analyzes gene gain and loss, and generates draft metabolic networks for comparison across genomes. Other functions of ITEP include analysis of functional domains, identification of core and variable genes and gene regions, and the use of closely related genomes to fix inaccurate annotations caused by gene fragmentation and sequencing errors (Benedict, Henriksen, Metcalf, Whitaker, & Price, 2014). Our use of ITEP

has only extended to examining the broad differences in genes across our constructed database of *Desulfovibrio* sp. to observe how many genes are conserved and are unique to our isolate (Table 2). Of the 14,758 genes analyzed, there are approximately 856 conserved across all organisms and 336 unique to 1L. Of those unique genes, there are 109 defined as "unknown function" according to ITEP's annotation (making up roughly a third of the unique 1L genes). Defined genes unique to 1L include some phage proteins, CRISPR elements, and ABC transport proteins for nickel. The CRISPR elements 1L shares with some of the other related organisms include *cas1, cas2,* and *cas4*. The other elements are unique to 1L in this particular comparison (*csd2, cas3, cas5,* associated protein tm1801, and HD domain-containing protein). Future analysis will dive deeper into this data and examine the evolutionary relatedness of 1L to its other *Desulfovibrio* neighbors.

A 16S rRNA maximum likelihood phylogenetic tree was constructed using the members of the *Desulfovibrio* sp. ITEP database to illustrate evolutionary relatedness (Figure 11).

Conclusions and Future Direction

The successful enrichment of PPL sediments resulted in the isolation of a sulfate-reducing bacterium and a fermenter under anaerobic conditions. Physiological experimentation has helped us understand isolate 1L's metabolic capabilities, with this isolate showing significant tolerance to elevated aqueous sulfide concentrations and demonstrating sensitivity to salinity. Our observations of isolate 1L's genome highlight its potential to play a key role in the cycles of carbon, sulfur, and nitrogen in the PPL region. Expanding our studies using genomic data as a reference is vital to understanding these

roles. The presence of genome identified enzymes for DNRA should be confirmed through experiment. Doing so would give validity to the suggested relationship 1L has with the nitrogen cycle and potentially uncover other relationships. The identity of fermentation products using high performance liquid chromatography and gas chromatography should be done in order to investigate 1L's central metabolism. In addition to this, the experiment for inducing bacteriophage should be revisited and adjusted to support the genomic evidence of bacteriophage. When thinking about how 1L is responding to environmental pressures like oxygen and saline, future studies should attempt to link flagellar movement with some of the functions of chemotaxis proteins that the genome has.

Considering the evidence of multiple CRISPRs, the detected spacer sequences should be aligned to detected phage genes in an attempt to derive a match. With CRISPR-associated genes identified, the next step in experimentation would be to investigate their organization in the operon. Knowing this information would allow us to properly classify 1L into a CRISPR/Cas system and subtype, make further claims about its relatedness to other CRISPR containing *Desulfovibrio* species, and determine if the element is active. The data uncovered so far in ITEP analysis shows how different our isolate is from its closest neighbors and offers some exciting directions for future investigation into the unique genes of unknown function. Additional analysis of this microbe's genome will allow for further hypotheses to be developed and tested, enabling inferences to be made regarding interactions between such SRB and other community members in PPL sediments and pore waters. Identifying these relationships is vital to understanding isolate 1L's place in the thermodynamic ladder and how it adds to the idea of competitive exclusion (Bethke, Sanford, Kirk, Jin, & Flynn, 2011a) (Figure S4). In particular, the relationship between SRBs

and methanogens is highly relevant to this study and should be the next aim of this research. Comprehending this would give insight to biogeochemical cycling in freshwater environments and allow larger claims to be made concerning the region. Additionally, scaling up our model of microbial influence may allow us to apply this knowledge to other systems around the world.

Acknowledgments

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Without the efforts and contributions of those involved, my work would not have been possible.

List of Figures and Tables

Supplemental Figures

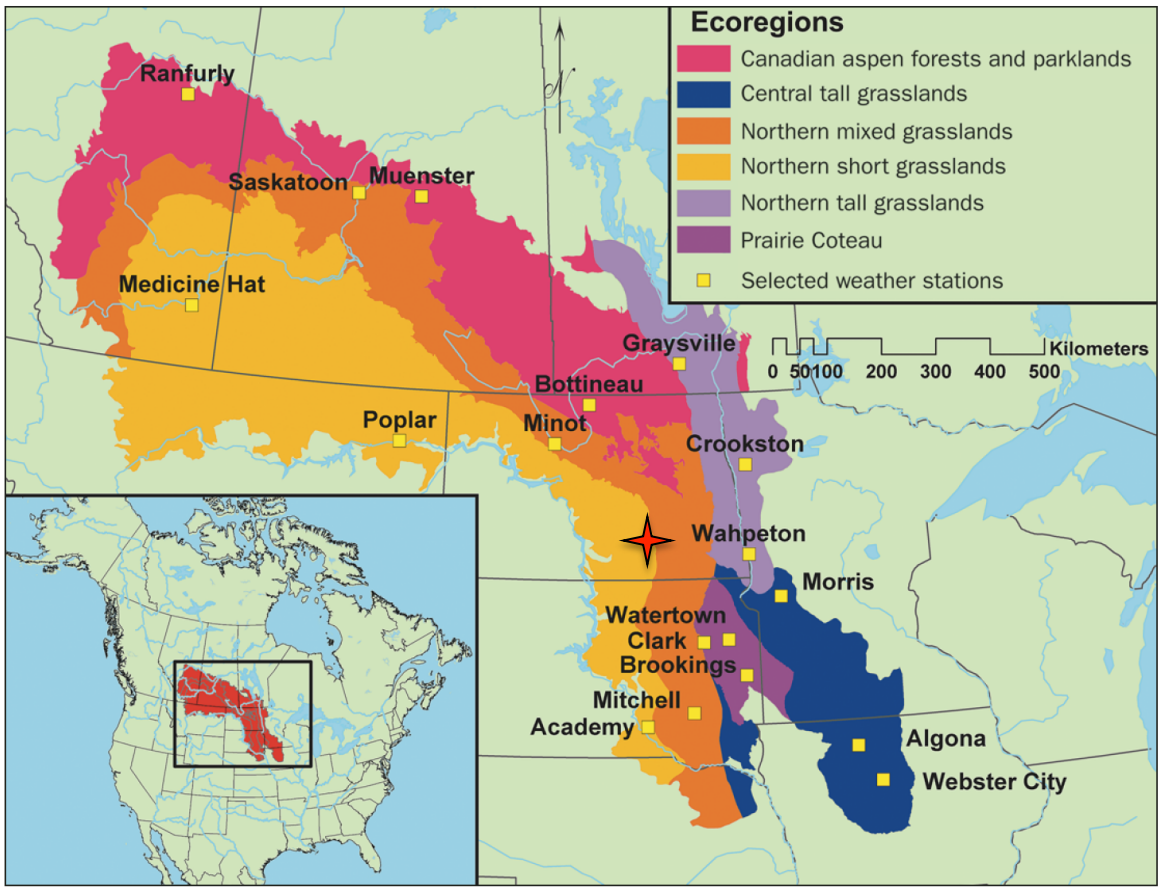


Figure 1. The Prairie Pothole Lake region is a natural, freshwater wetland ecosystem that contains millions of small lakes and covers about 750,000 km^2 in North America. Each lake has its own unique biochemical properties. Samples in this study were taken at a lake near Jamestown, ND marked by the red star.

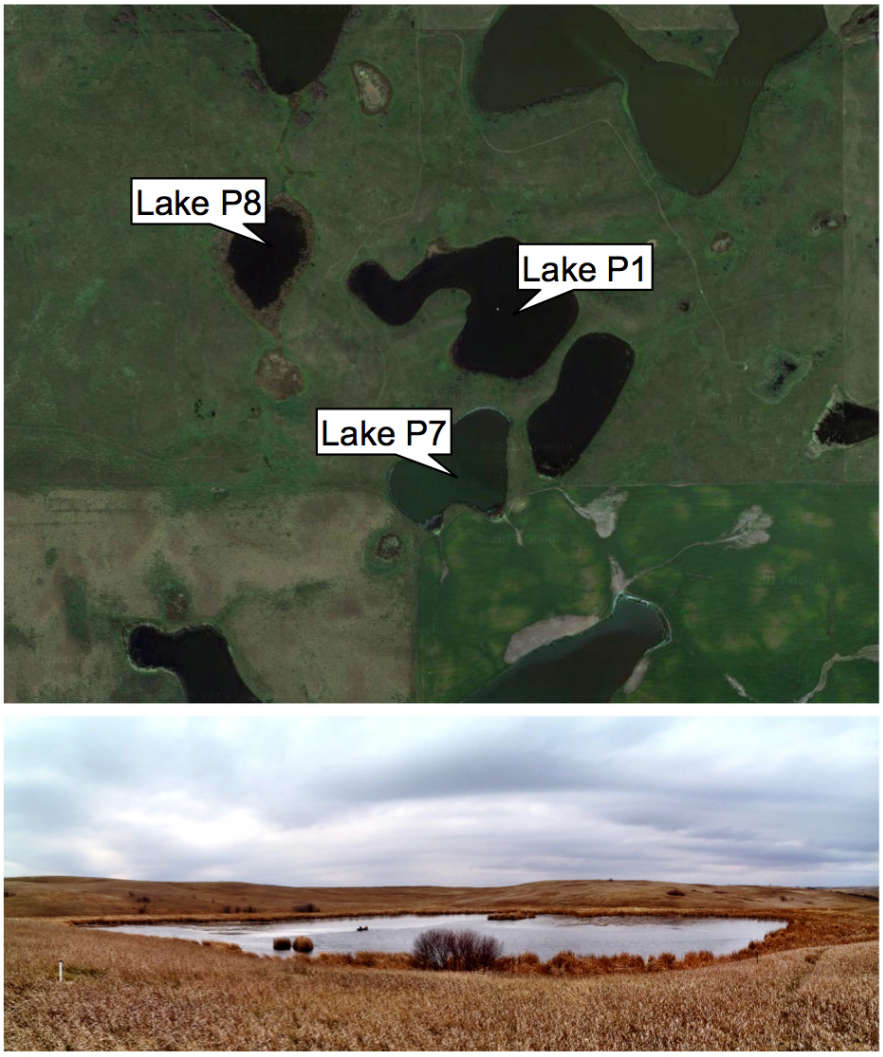


Figure 2. Prairie Pothole Lake P1. Enrichment sediment and water samples were taken from lake P1 and water from lake P7 was concentrated for the filtering and extraction of DOC.

Figure 3. Experimental setup of Isolation pipet bulbs. Bulbs were pre-inoculated with culture, filled with warm media, amended with electron donor and acceptor, and sealed shut with zip ties. Bulbs were stored in the anaerobic chamber in a 40ºC incubator.

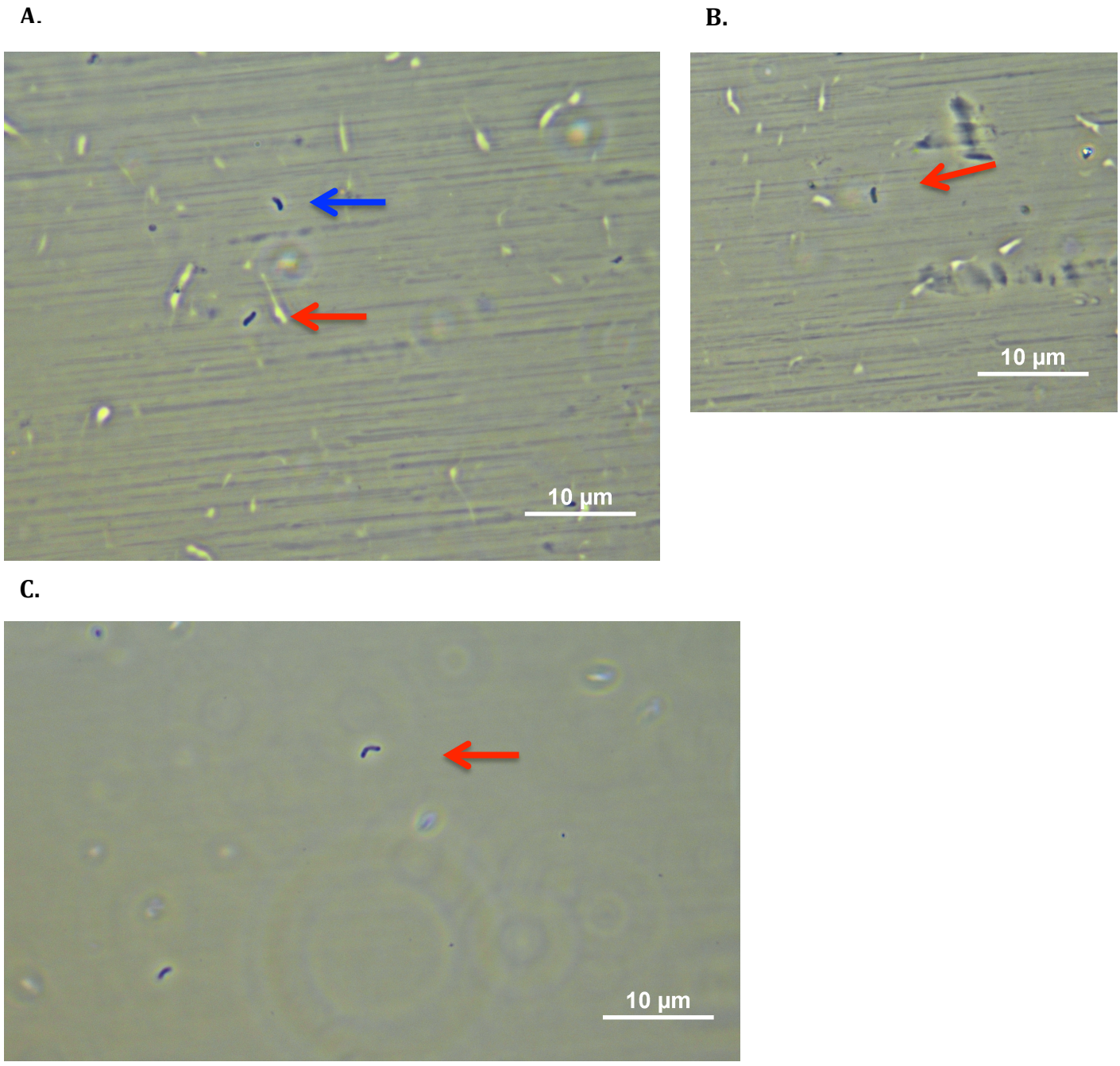


Figure 4. Bright field images of isolate 1L. These images were taken using a Nikon Eclipse Ci-S/Ci-L microscope at magnification 1000X. The length of the microbes in each individual image is noted: **A.** blue arrow 1.51µm red arrow 1.61µm **B.** 1.61µm **C.** 2.16µm

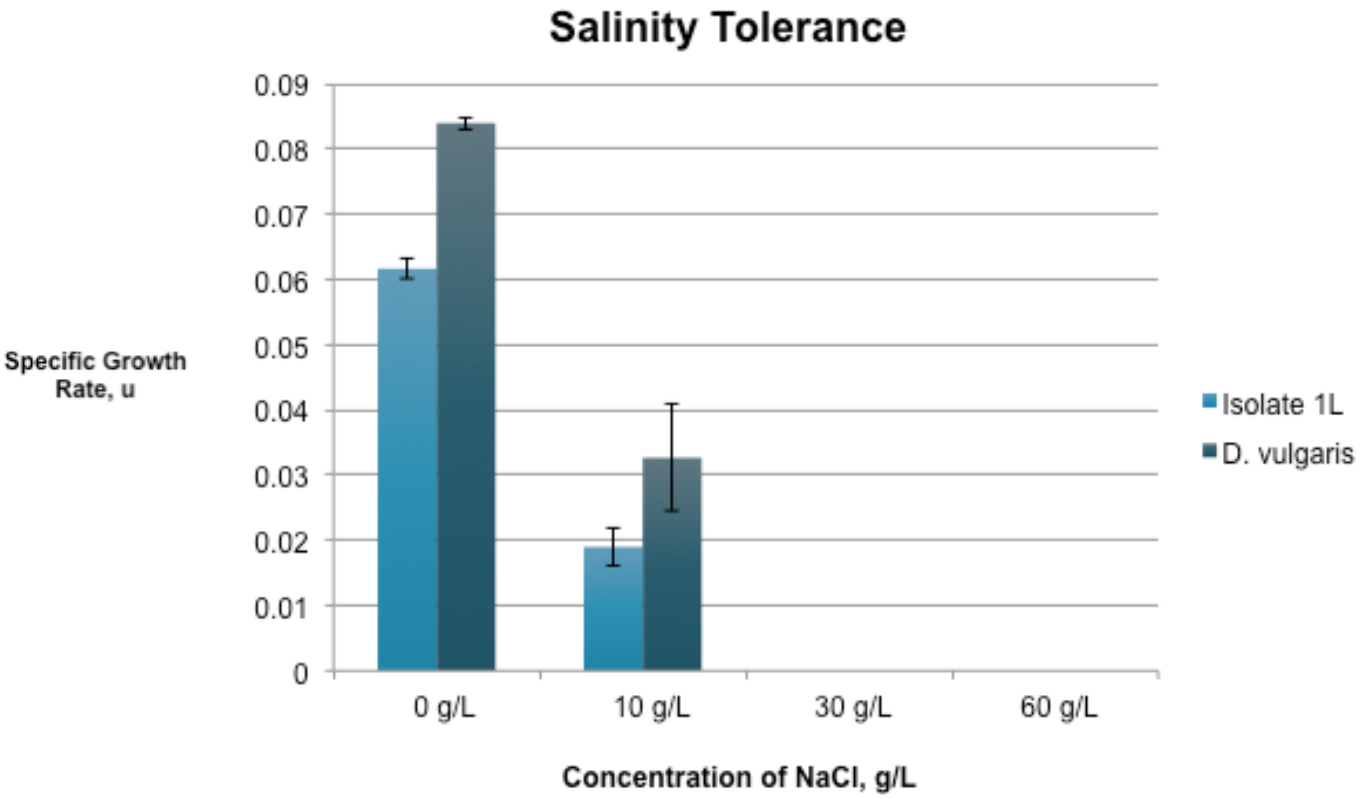


Figure 5. Salinity Tolerance of Isolate 1L. Optical density data from growth curves performed for each concentration in triplicate was collected over 315 hours. The specific growth rates for each curve were calculated, averaged, standard deviation determined, and plotted against concentration of saline. *Desulfovibrio vulgaris* was used as a representative organism for comparison. No growth was observed at concentrations above 10 g/L.

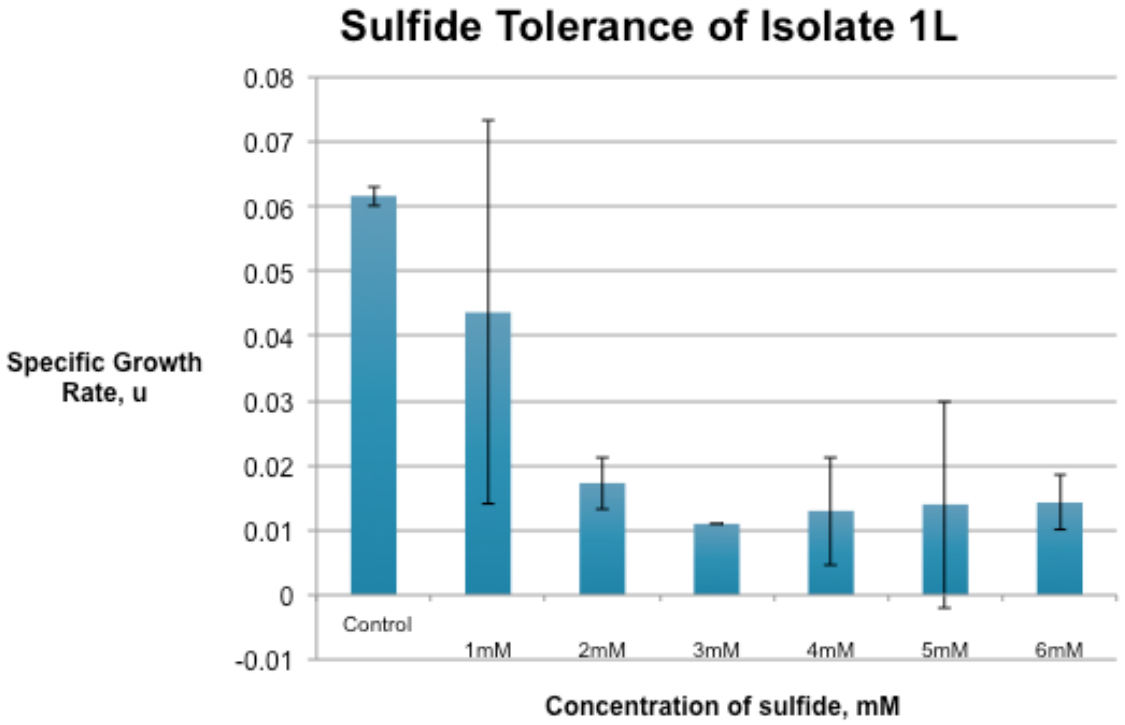


Figure 6. Sulfide Tolerance of Isolate 1L. Optical density data from growth curves performed for each concentration in triplicate was collected over 200 hours. Specific growth rates for each curve were calculated, averaged, standard deviation determined, and plotted against the corresponding concentration of sulfide.

Electron Acceptor	Electron Donor	Growth?
Sulfate	Butyrate	No
Sulfate	Formate	No
Sulfate	Propionate	No
Sulfate	Acetate	Minimal/No
Sulfate	Lactate	Yes
Sulfate	Ethanol	Yes
Thiosulfate	Lactate	Yes

Table 1. Substrate Utilization of Isolate 1L. The sodium salts of each substrate (excluding ethanol) were used to amend cultures at a concentration of 20mM. Growth was assessed by comparing OD600 readings to a standard 1L culture on sulfate and lactate in freshwater media.

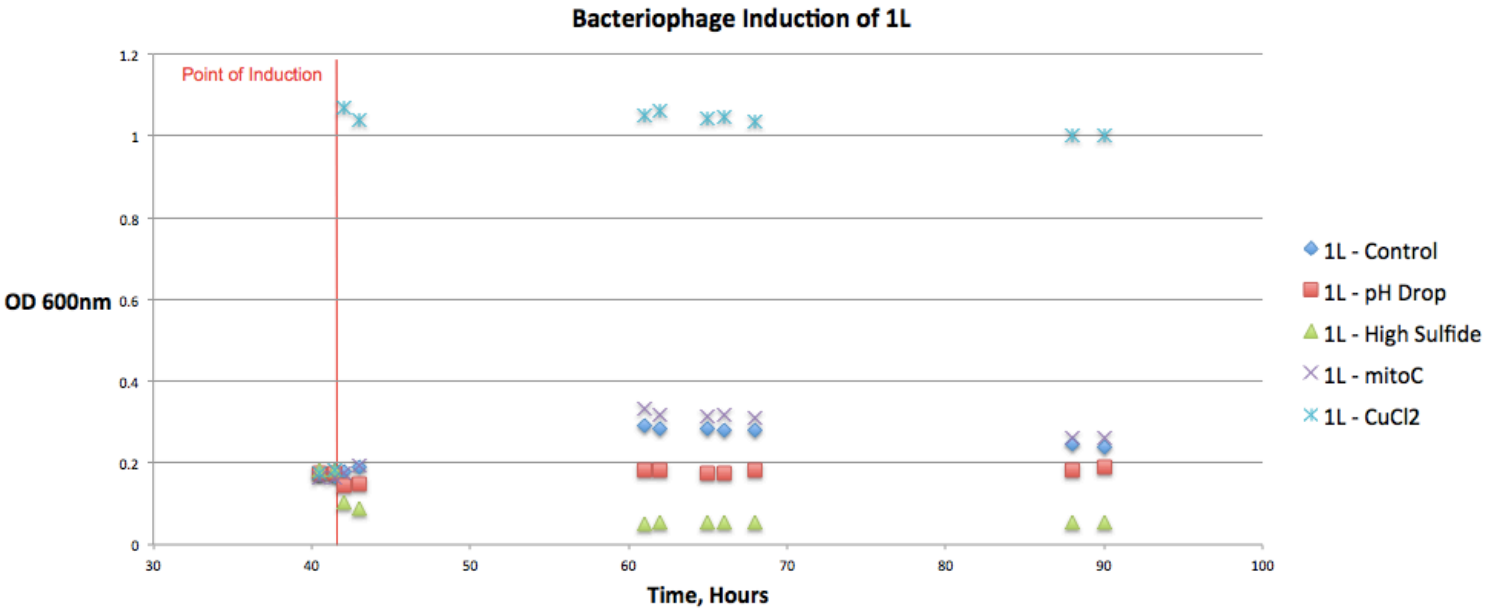


Figure 7. Bacteriophage Induction of 1L. Isolate 1L was induced with a variety of cellular stressors to detect the presence of bacteriophage. Cells were induced around 42 hours after inoculation, which is a time before their projected peak growth. The data do not show evidence of the presence of phage.

Scaffold	Start site	Stop site	Length, base pairs	# of Spacers
scaffold_61_2	31837	32931	1094	16
scaffold_103_1	6608	7422	814	12
scaffold_103_2	7655	7949	294	4
scaffold_152_1	98	10593	10495	158
scaffold_157_1	625	879	254	3

Figure 8. CRISPR sequences detected by CRISPRFinder. The nucleotide data file from genomic sequencing data for 1L was used as an input into the CRISPRFinder website and default settings used. This table is a summary of the confirmed systems by the program.

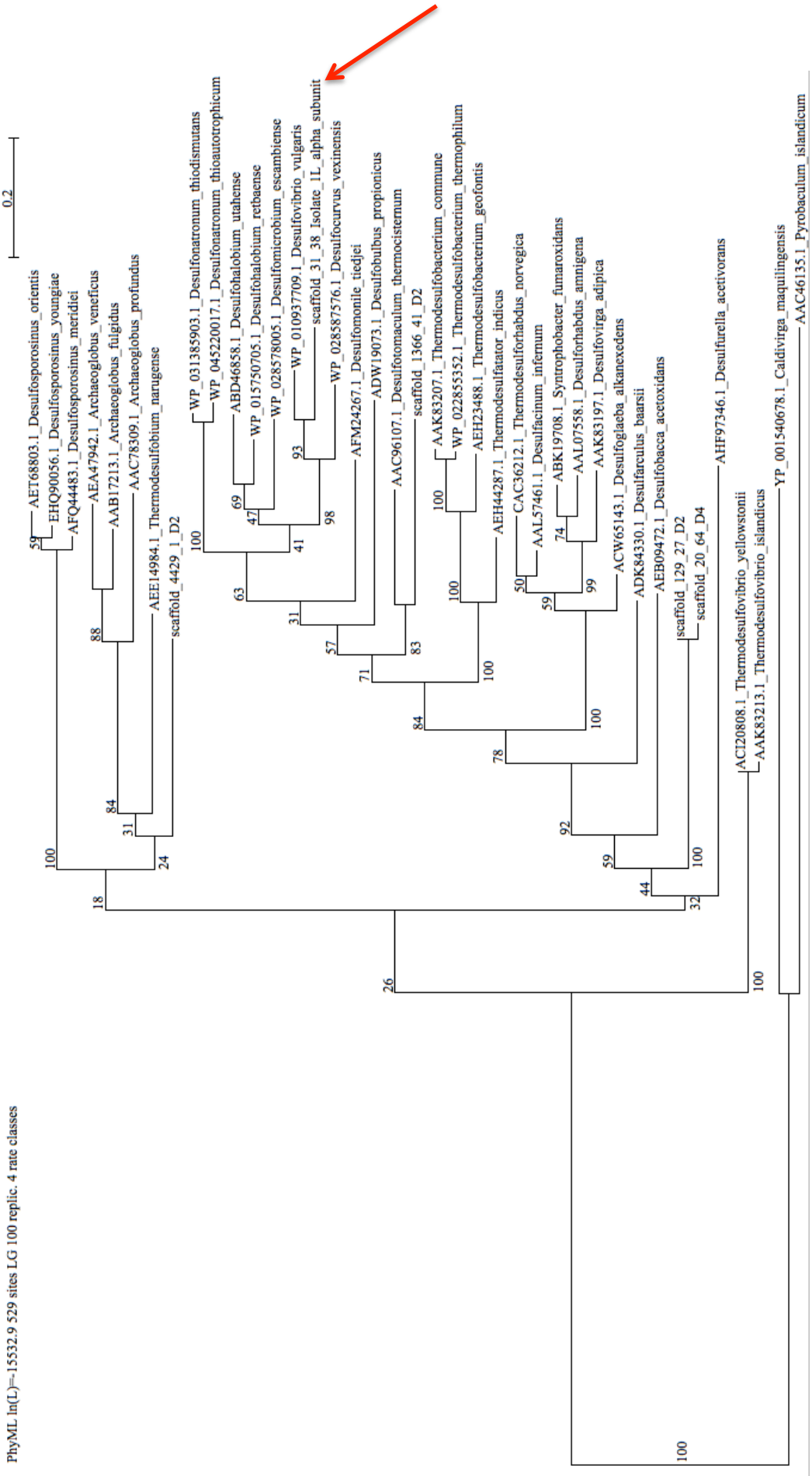
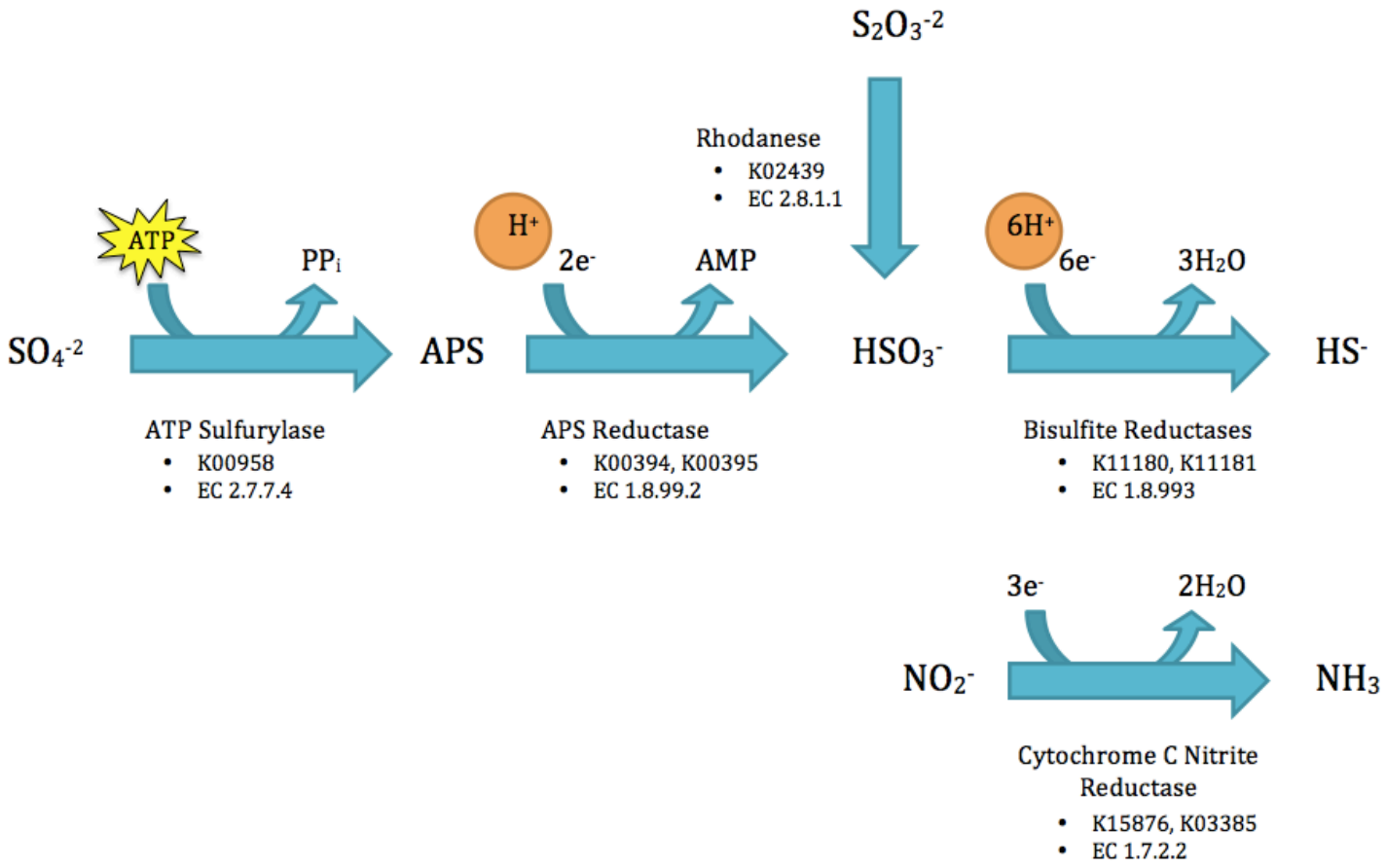


Figure 9. PhyML phylogenetic tree for *dsrA* gene sequences. A maximum likelihood phylogenetic tree was built in the tree-building program Seaview. Translated amino acid sequences for the *dsrA* genes were pulled from the SILVA database, metagenomic data, and 1L's translated amino acid sequences for DsrA and DsrB derived from genome data. The PhyML program was used and bootstrapped 100 times. Bootstrap values are indicated and accession numbers next to their associated organisms. The red arrow indicates the position of 1L's *dsrA* gene.



Gene	Scaffold_Gene	KO number	EC number
ATP Sulfurylase	scaffold_0_29	K00958	2.7.7.4
APS Reductase	subunit A scaffold_12_92 subunit B scaffold_12_93	K00394 K00395	1.8.99.2
Bisulfite Reductases	α scaffold_31_38 β scaffold_31_37	K11180 K11181	1.8.993
Rhodanese (thiosulfate sulfurtransferase)	scaffold_3_6	K02439	2.8.1.1
Cytochrome C Nitrite Reductase	scaffold_27_10 scaffold_27_11	K15876 K03385	1.7.2.2

Figure 10. Sulfate Reduction in Microorganisms. A flowchart portraying the process of sulfate reduction with added features. The rhodanese enzyme (also known as thiosulfate sulfurtransferase) allows for a more efficient reduction without the need of an energy input. We also hypothesize a mechanism for disposing of excess reductant in environments with abundant electron donor (DOC) by means of dissimilatory nitrate reduction to ammonia (DNRA) with the cytochrome c nitrite reductase enzyme. The table below provides additional information about the enzymes.

ITEP Statistics	
Total genes among 11 organisms	14,758
Total genes in common	856
Genes unique to 1L	336
1L unique genes with unknown function	109

Table 2. ITEP Statistics concerning 1L. The program ITEP (An integrated toolkit for exploration of microbial pan-genomes) was used to observe genes conserved across related organisms. 1L's genome was compared to a constructed database of ten other *Desulfovibrio* sp.

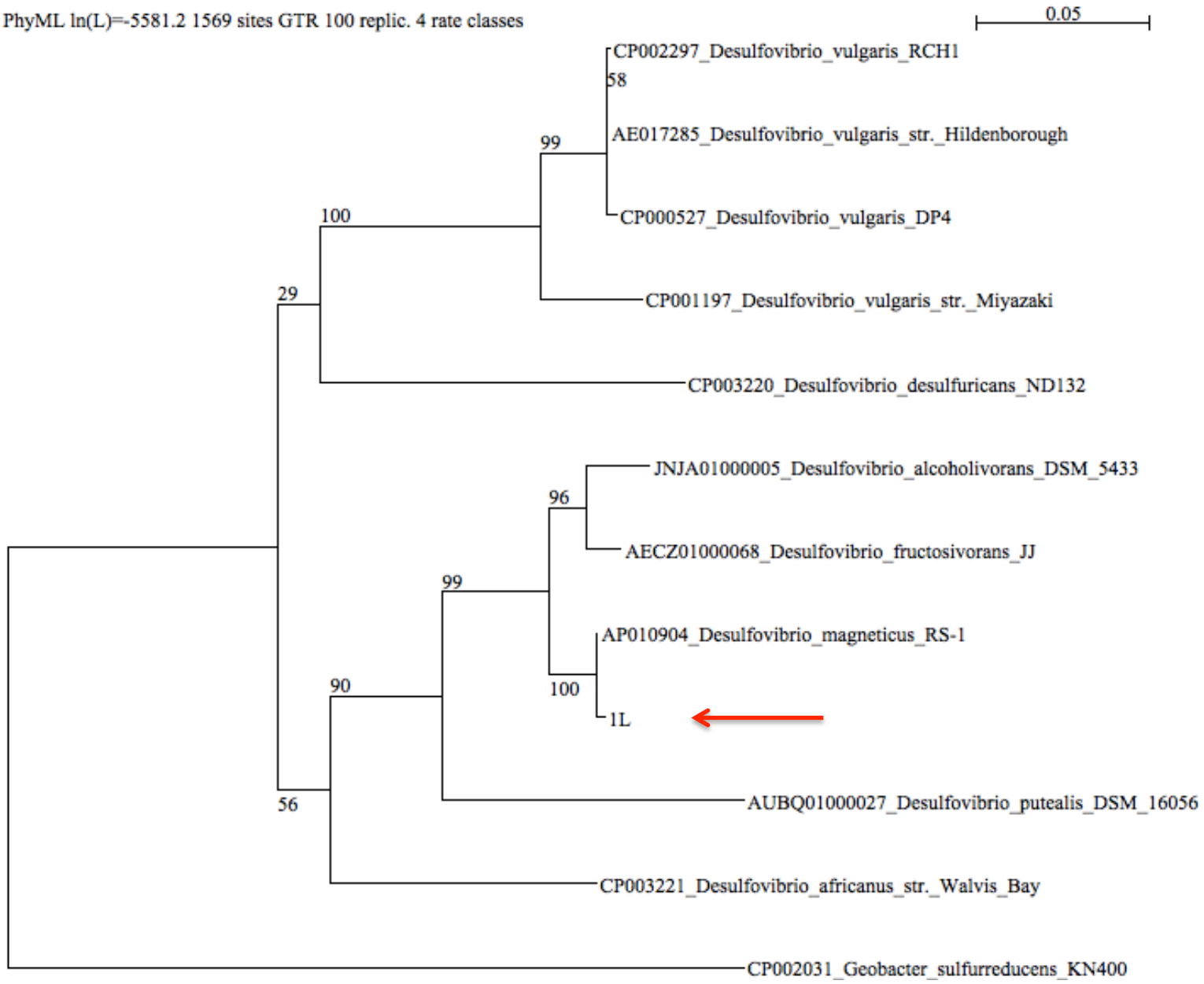


Figure 11. Maximum likelihood phylogenetic tree. Using the *Desulfovibrio* sp. from the constructed ITEP database (except for the tree root) and isolate 1L, a maximum likelihood phylogenetic tree was built in the tree-building program Seaview. 16S rRNA gene sequences were pulled from the SILVA database and 1L's 16S gene was derived from DNA sequencing. The PhyML program was used and bootstrapped 100 times. Bootstrap values are indicated and accession numbers next to their associated organisms. A red arrow emphasizes the placement of 1L.

Supplemental Figures

Fresh Water (FW) Media

Ingredient/Chemical	1 Liter
Milli-Q Deionized H_2O	800 mL
$NaHCO_3$	2.50 g
$NaH_2PO_4 \bullet H_2O$	0.60 g
NH_4Cl	0.25 g
KCl	0.10 g
Vitamin mix	10 mL
Mineral mix	10 mL
Ferrous Sulfate**	0.05 g
Agarose**	15 g

**Added when Isolation Media is prepared

YPD Media

Ingredient/Chemical	500 mL
$NaHCO_3$	2.00 g
KCl	0.50 g
NH_4Cl	0.50 g
Na_2HPO_4	0.25 g
Cysteine	0.50 g
Sodium Citrate	0.40 g
$CaCl_2 \bullet 2H_2O$	0.20 g
Yeast Extract	1.00 g
Peptone/Tryptone	1.00 g
Dextrose (D-glucose)	20mM 1.80 g
Mineral Mix	5 mL
Vitamin Mix	5 mL

Figure S1. Recipe for Fresh Water and YPD Media. Contents are added to a volume of deionized water and final volume adjusted. Media is then driven anaerobic for 45 minutes by a gas mixture of N_2:CO_2 [95:5], pH adjusted to ~7-7.1, and then headspace degassed for 10 minutes. Media is then brought into the anaerobic chamber to be dispersed to serum bottles, which are capped and sealed before being autoclaved on a liquid cycle with a 20-minute sterilization time.

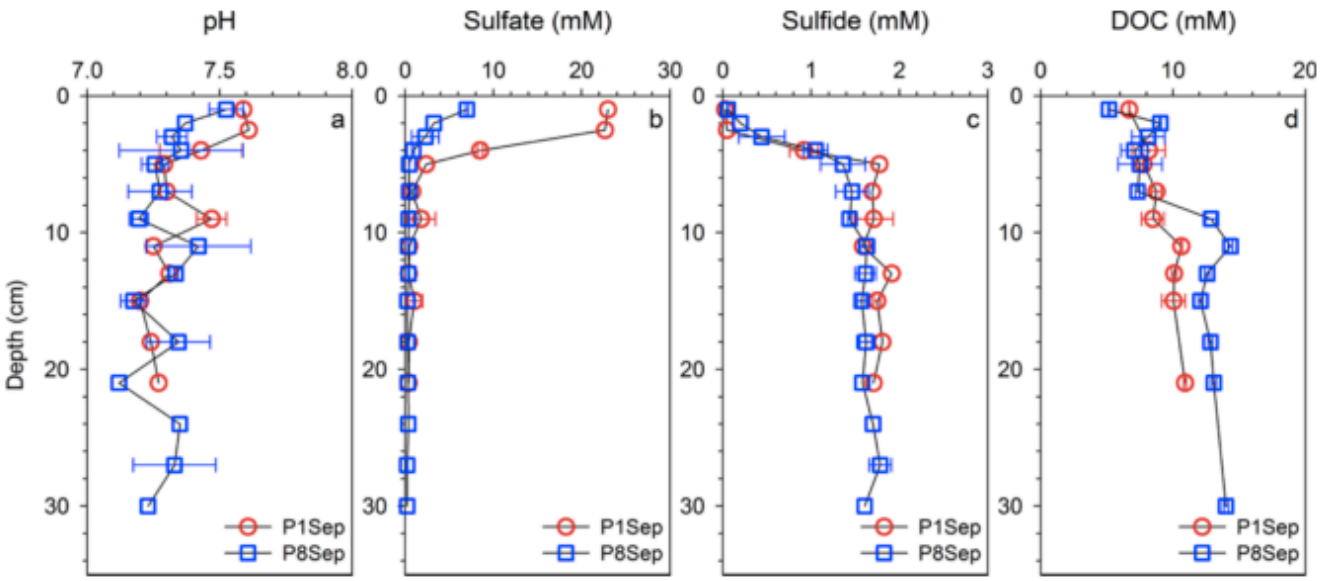


Figure S2. Depth profiles of pH, sulfate, sulfide, and DOC for P1Sep and P8Sep sediment porewater samples. The sediment–water interface is dictated by "0cm". Error bars represent one standard deviation of duplicate samples; where absent, only one sample was analyzed. DOC data were averaged from values reported by Ziegelgruber, 2011. (Zeng et al., 2013)

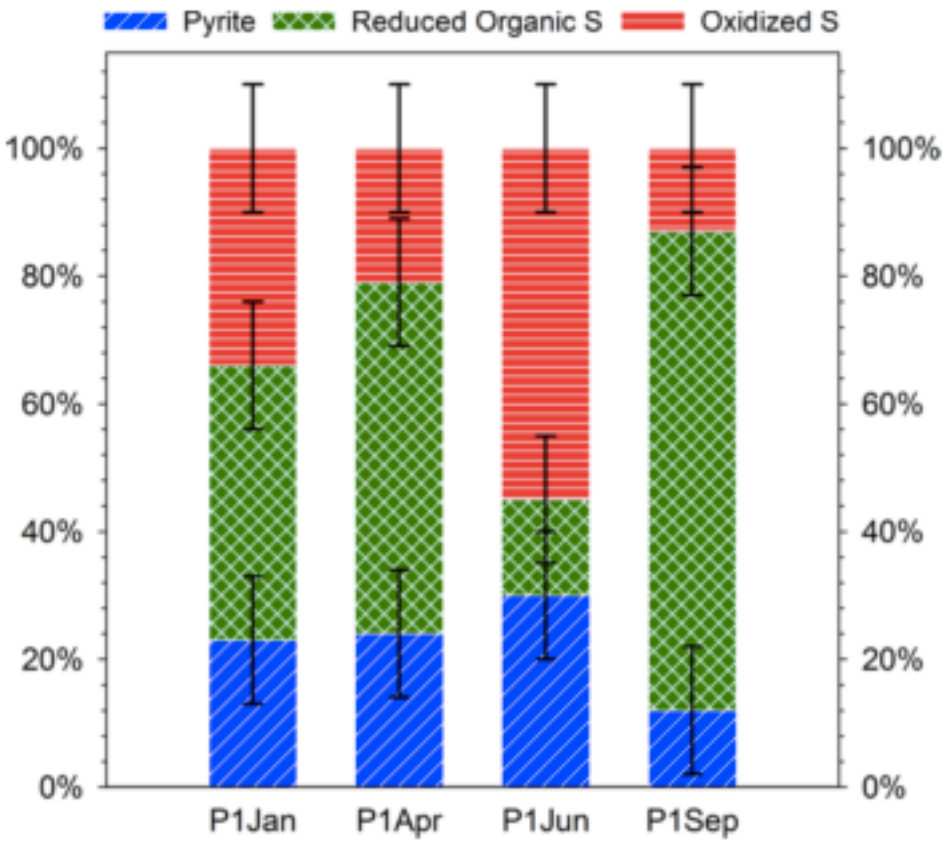


Figure S3. Fractional contribution of pyrite, reduced organic S, and oxidized S in the P1 sediment samples with the sum of all S species normalized to 100 mol %. (Zeng et al., 2013)

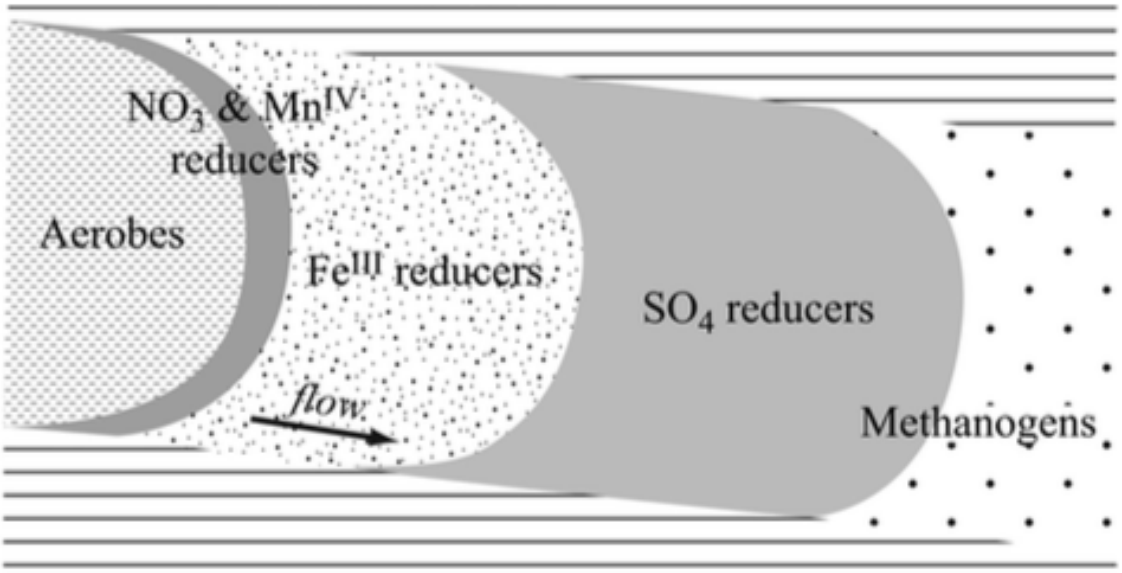


Figure S4. Microbiological zoning as envisioned to occur in a pristine aquifer, showing arrangement of zones according to a thermodynamic ladder of electron accepting processes. We believe the PPL microenvironment functions similarly through the idea of competitive exclusion where thermodynamics control which microorganisms are able to access electron donors according to an energetic hierarchy of electron accepting processes (Bethke, Sanford, Kirk, Jin, & Flynn, 2011).

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